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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	8449-115-999	*Total Pages*	64
First Named Inventor or Application Identifier			
Pramod K. Srivastava			
Express Mail Label No.	EL 501 633 878 US		

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

1. ☒ Fee Transmittal Form
   Submit an original, and a duplicate for fee processing)
2. ☒ Specification [*Total Pages* 36]
   (preferred arrangement set forth below)
   - -Descriptive title of the Invention
   - -Cross Reference to Related Applications
   - -Statement Regarding Fed sponsored R&D
   - -Reference to Microfiche Appendix
   - -Background of the Invention
   - -Brief Summary of the Invention
   - -Brief Description of the Drawings *(if filed)*
   - -Detailed Description of the Invention (including drawings, *if filed*)
   - -Claim(s)
   - -Abstract of the Disclosure
3. ☐ Drawing(s) *(35 USC 113)* [*Total Sheets*]
4. ☒ Oath or Declaration [*Total Sheets* 6]
   a. ☐ Newly executed (original or copy)
   b. ☒ Copy from a prior application (37 CFR 1.63(d))
   (for continuation/divisional with Box 17 completed)
   [Note Box 5 below]
   i. ☐ DELETION OF INVENTORS(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33 (b).
5. ☒ Incorporation By Reference *(useable if Box 4b is checked)*
   The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

ADDRESS TO: **Assistant Commissioner for Patents**
Box Patent Application
Washington, DC 20231

6. ☐ Microfiche Computer Program *(Appendix)*
7. ☐ Nucleotide and/or Amino Acid Sequence Submission
   (if applicable, all necessary)
   a. ☐ Computer Readable Copy
   b. ☐ Paper Copy (identical to computer copy)
   c. ☐ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))
9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
   (when there is an assignee)
10. ☐ English Translation Document *(if applicable)*
11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
12. ☒ Preliminary Amendment
13. ☒ Return Receipt Postcard (MPEP 503)
    (Should be specifically itemized)
14. ☒ Copy of two Verified Statements Claiming Small Entity Status filed in prior Application Serial No. 08/315,892, status still proper and desired
15. ☐ Certified Copy of Priority Document(s)
    (if foreign priority is claimed)
16. ☒ Other: Copy of Revocation and Power of Attorney; copy of Supplemental Declaration and Power of Attorney filed in prior Application Serial No. 08/315,892.

17. **If a CONTINUING APPLICATION,** *check appropriate box and supply the requisite information:*
☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application Serial No. 09/489,218, filed January 21, 2000, which is a Continuation of prior application Serial No. 09/061,365 filed April 16, 1998, which is a divisional of prior application Serial No. 08/315,892 filed September 30, 1994.

18. CORRESPONDENCE ADDRESS

☒ Customer Number or Bar Code Label 20583 *(Insert Customer No. or Attach bar code label here)* or ☐ Correspondence address below

NAME					
ADDRESS					
CITY		*STATE*		*ZIP CODE*	
COUNTRY		*TELEPHONE*		*FAX*	

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Box Patent Application, Washington, DC 20231.

Express Mail No.: EL 501 633 878 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Prior application: Examiner To be assigned

Art Unit 1648

Assistant Commissioner for Patents
Box PATENT APPLICATION
Washington, DC 20231

Sir:

This is a request for filing a ☒ continuation ☐ divisional application under 37 CFR § 1.53(b), of pending prior application no. 09/489,218 filed on January 21, 2000

of Pramod K. Srivastava
(inventor(s) currently of record in prior application)

for Immunotherapeutic Stress Protein-Peptide Complexes Against Cancer
(title of invention).

1. ☒ The filing fee is calculated below, taking into account the Preliminary Amendment submitted herewith:

PATENT APPLICATION FEE VALUE

TYPE	NO. FILED	LESS	EXTRA	EXTRA RATE	FEE
Total Claims	51	-20	31	$22.00 each	682.00
Independent	5	-3	2	$82.00 each	164.00
			Basic Fee		690.00
			Multiple Dependency Fee If Applicable ($270.00)		270.00
			Total		1,806.00
			50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern		- 903.00
			Total Filing Fee		$ 903.00

2. ☒ Please charge the required fee to Pennie & Edmonds LLP Deposit Account No. 16-1150. A copy of this sheet is enclosed.

3. ☒ Amend the specification by inserting on page 1, under the title, the following sentence: --This is a continuation of application no. 09/489,218 filed January 21, 2000, which is a continuation of application no. 09/061,365 filed April 16, 1998, now U.S. Patent No. 6,017,544, which is a division of application no. 08/315,892 filed September 30, 1994, now U.S. Patent No. 5,750,119, each of which is incorporated by reference herein in its entirety.--

4a.	☐	Transfer the drawings from the prior application to this application and abandon the prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file.
4b.	☐	New formal drawings are enclosed.
4c.	☐	Informal drawings are enclosed.
5a.	☐	Priority of application no. ϕϕ filed on ϕϕ in ϕϕ is claimed under 35 U.S.C. §119.
5b.	☐	The certified copy has been filed in prior application no. ϕϕ, filed ϕϕ.
6.	☒	The prior application is assigned of record to Mount Sinai School of Medicine of New York University.
7a.	☒	The Power of Attorney appears in the original papers in the prior application no. 08/315,892, filed September 30, 1994; a copy of the Power of Attorney is submitted herewith. A copy of the Supplemental Declaration and Power of Attorney filed in prior Application Serial No. 08/315,892 is also submitted herewith.
7b.	☐	Since the Power of Attorney does not appear in the original papers, a copy of the Power in prior application no. ϕϕ, filed ϕϕ is enclosed.
8.	☐	This application contains nucleic acid and/or amino acid sequences required to be disclosed in a Sequence Listing under 37 CFR §§1.821-1.825. It is requested that the Sequence Listing in computer readable form from prior application no. ϕϕ, filed on ϕϕ be made a part of the present application as provided for by 37 C.F.R. §1.821(e). The sequences disclosed therein are the same as the sequences disclosed in this application. A copy of the paper Sequence Listing from application no. ϕϕ is enclosed.
9.	☐	The undersigned states, under 37 C.F.R. §1.821(f), that the content of the enclosed paper Sequence Listing from application no. ϕϕ is the same as the content of the computer readable form submitted in application no. ϕϕ.

10. ☒ Other Enclosures: Preliminary Amendment; copy of Revocation and Power of Attorney for Application Serial No. 08/315,892; and copy of two Verified Statements Claiming Small Entity Status for Application Serial No. 08/315,892,

Respectfully submitted,

Date: September 8, 2000

Adriane M. Antler 32,605
Adriane M. Antler (Reg No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, NY 10036
Tel. No. (212) 790-9090

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: ☒ Application of: PRAMOD K. SRIVASTAVA
☐ Patent of:

☒ Serial No.: 08/315,892 — Group Art Unit: 1205
☐ Patent No.:

☒ Filed: September 30, 1994 — Examiner: Goldberg, Jerome D.
☐ Issued:

For: IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER — Attorney Docket No.: 8449-008

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) and 1.27(c)] - Small Business Concern

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I hereby declare that I am

☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act in behalf of the concern identified below:
Name of concern ANTIGENICS, INC.
Address of concern 30 Rockefeller Plaza, Suite 4220
New York, New York 10112

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the person employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern and/or there is an obligation under contract or law by the inventor(s) to convey rights to the small business concern with regard to the invention, entitled IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER by inventor PRAMOD K. SRIVASTAVA described in

☐ the specification filed herewith
☒ application serial no. 08/315,892 filed September 30, 1994
☐ patent no. issued

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

FULL NAME MOUNT SINAI SCHOOL OF MEDICINE OF THE CITY UNIVERSITY OF NEW YORK
ADDRESS One Gustave L. Levy Place
New York, New York 10029

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28 (b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, or any patent to which this verified statement is directed.

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1155 Avenue of the Americas
New York, N.Y. 10036-2711

Direct Telephone calls to:
PENNIE & EDMONDS
(212) 790-9090

Name of person signing GARO H. ARMEN, PH.D.
Title of person other than owner President and Chief Executive Officer
Address of person signing 30 Rockefeller Plaza, Suite 4220
New York, New York 10112

Signature _____ Date Jan 15 1996

***NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: ☒ Application of: Pramod K. Srivastava
☐ Patent of:

☒ Serial No.: 08/315,892 — Group Art Unit: 1205
☐ Patent No.:

☒ Filed: September 30, 1994 — Examiner:
☐ Issued:

For: IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER — Attorney Docket No.: 8449-008

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) and 1.27(d)] - Nonprofit Organization

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

Sir:

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of organization **Mount Sinai School of Medicine of the City University of New York**
Address of organization **One Gustave L. Levy Place New York, New York 10029**

Type of organization
☒ University or other institution of higher education
☐ Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
☐ Nonprofit scientific or educational under statute of state of the United States of America
(Name of state _____)
(Citation of statute _____)
☐ Would qualify as tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)) if located in the United States of America.
☐ Would qualify as nonprofit scientific or educational under statute of state of the United States of America if located in the United States of America
(Name of state _____)
(Citation of statute _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled **IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER** by inventor(s) **PRAMOD K. SRIVASTAVA** described in

☐ the specification filed herewith
☒ application serial no. 08/315,892 filed September 30, 1994
☐ patent no. issued

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization identified above and/or there is an obligation under contract or law by the inventor(s) to convey rights to the nonprofit organization identified above with regard to the invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

FULL NAME ANTIGENICS, INC.
ADDRESS 30 Rockefeller Plaza, Suite 4220
New York, New York 10112
☐ INDIVIDUAL ☒ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

FULL NAME _____
ADDRESS _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28 (b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, or any patent to which this verified statement is directed.

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Direct Telephone calls to:
PENNIE & EDMONDS
(212) 790-9090

Name of person signing Beth Essig, Esq.
Title of person other than owner Vice President & Associate General Counsel
Address of person signing Mount Sinai ~~Medical Center~~ School of Medicine, 19 East 98th Street, Room 7A, Box 1009, New York, New York 10029-6574

Signature _____ Date 4/11/95

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Express Mail No: EL 501 633 878 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Pramod K. Srivastava

Application No.: To be Assigned | Art Unit: To be Assigned

Filed: On Even Date Herewith | Examiner: To be Assigned

For: Peptides From Stress Protein-peptide Complexes (as amended) | Attorney Docket No.: 8449-115-999

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Pursuant to 37 C.F.R. § 1.111, please consider the following amendments and remarks prior to examination of the above-identified application on the merits.

IN THE SPECIFICATION

Please delete on page 1, in the title "IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER" and insert --PEPTIDES FROM STRESS PROTEIN-PEPTIDE COMPLEXES--.

IN THE CLAIMS

Please amend the claims as follows:

Cancel claims 2 to 18 without prejudice.

Please add the following new claims:

19. (New) A composition comprising an amount of a purified population of peptides, wherein said purified population of peptides is produced by a method comprising the steps of:

a) purifying a population of non-covalently associated stress protein-peptide complexes from mammalian tumor cells;

b) releasing the peptides from said population of complexes; and

c) recovering the released population of peptides.

20. (New) A purified peptide that is present as a non-covalent complex with a stress protein in a mammalian tumor cell.

21. (New) A purified peptide consisting of the amino acid sequence of a peptide that is present as a non-covalent complex with a stress protein in a mammalian tumor cell.

22. (New) The composition of claim 19 further comprising a cytokine.

23. (New) The composition of claim 22 wherein said cytokine is selected from the group consisting of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IFNα, IFNβ, IFNγ, TNF∝, TNFβ, G-CSF, GM-CSF, and TGF-β.

24. (New) The composition of claim 19 wherein the peptides are released from said population of complexes by a method comprising placing said population of complexes in the presence of adenosine triphosphate, low pH, or both.

25. (New) The composition of claim 19, wherein said mammalian tumor cells are human cells.

26. (New) The composition of claim 19 wherein said mammalian tumor cells are from a tumor selected from the group consisting of melanocarcinoma, hepatocarcinoma, and renal cell carcinoma.

27. (New) The composition of claim 19 wherein said tumor cells are from a metastasis.

28. (New) The composition of claim 19, wherein said tumor cells have been proliferated in vivo.

29. (New) The composition of claim 19, wherein said tumor cells have been proliferated in vitro.

30. (New) The composition of claim 19, wherein the stress protein is a member of a stress protein family selected from the group consisting of hsp60, hsp70, and hsp90.

31. (New) The composition of claim 19, wherein the stress protein is gp96.

32. (New) The peptide of claim 20 or 21, wherein said mammalian tumor cell is a human cell.

33. (New) The peptide of claim 20 or 21 wherein said mammalian tumor cell is from a tumor selected from the group consisting of melanocarcinoma, hepatocarcinoma, and renal cell carcinoma.

34. (New) The peptide of claim 20 or 21, wherein said tumor cell is from a metastasis.

35. (New) The peptide of claim 20, wherein said tumor cell has been proliferated in vivo.

36. (New) The peptide of claim 21, wherein said tumor cell has been proliferated in vitro.

37. (New) The peptide of claim 20 or 21, wherein the stress protein is a member of a stress protein family selected from the group consisting of hsp60, hsp70, and hsp90.

38. (New) The peptide of claim 20 or 21, wherein the stress protein is gp96.

39. (New) A method of making a composition comprising a population of peptides comprising:

a) purifying a population of stress protein-peptide complexes from mammalian tumor cells;

b) releasing a population of peptides from said population of complexes; and

c) recovering the released population of peptides.

40. (New) A method of making a purified peptide comprising:

a) purifying a population of stress protein-peptide complexes from mammalian tumor cells;

b) releasing a population of peptides from said population of complexes; and

c) purifying a peptide from the released population of peptides.

41. (New) The method of claim 39 wherein the peptides are released from said population of complexes by a method comprising placing said population of complexes in the presence of adenosine triphosphate, low pH, or both.

42. (New) The method of claim 40 wherein the peptides are released from said population of complexes by a method comprising placing said population of complexes in the presence of adenosine triphosphate, low pH, or both.

43. (New) The method of claim 39 or 40 wherein said mammalian tumor cells are human cells.

44. (New) The method of claim 39 or 40, wherein said mammalian tumor cells are from a tumor selected from the group consisting of melanocarcinoma, hepatocarcinoma, and renal cell carcinoma.

45. (New) The method of claim 39 or 40, wherein said mammalian tumor cells are from a metastasis.

46. (New) The method of claim 39 or 40, wherein said mammalian tumor cells have been proliferated in vitro.

47. (New) The method of claim 39 or 40, wherein said mammalian tumor cells have been proliferated in vivo.

48. (New) The method of claim 39 or 40, wherein the stress protein is a member of a stress protein family selected from the group consisting of hsp60, hsp70, and hsp90.

49. (New) The method of claim 39 or 40, wherein the stress protein is gp96.

50. (New) A composition comprising the purified peptide of claim 20 or 21 and a cytokine.

51. (New) The composition of claim 50 wherein said cytokine is selected from the group consisting of IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IFNα, IFNβ, IFNγ, TNF∝, TNFβ, G-CSF, GM-CSF, and TGF-β.

REMARKS

The title of the application has been amended to better reflect the claimed subject matter.

Claims 2-18 have been canceled without prejudice, and new claims 19-51 added, to more particularly point out and distinctly claim that which Applicant regards as the invention. The subject matter of the new claims is fully supported in the specification. In particular, support for the new claims is found in the specification as set forth in the chart below. No new matter is introduced.

New Claims	Support in Specification
19, 20, 21, 24, 39, 40, 41, 42	page 5, lines 23-27; page 6, lines 22-29; page 8, lines 9-18
22, 23, 50, 51	page 9, line 28 to page 10, lines 15
25, 32, 43	page 13, lines 24-26
26, 33, 44	page 15, lines 15-16
27, 34, 45	page 13, lines 14-17
28, 29, 35, 36, 46, 47	page 16, lines 30-35
30, 31, 37, 38, 48, 49	page 17, lines 17-19

CONCLUSION

Applicant respectfully requests that the above-made amendments be entered and made of record in the instant application. An early allowance is earnestly requested.

Respectfully submitted,

Date: September 8, 2000

Adriane M. Antler 32,605
Adriane M. Antler (Reg. No.)

PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, NY 10036-2711
(212) 790-9090

Enclosure

EXPRESS MAIL NO.: EL 501 633 878 US

PATENT APPLICATION
Attorney Docket No.: 8449-115-999

IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER

Field of the Invention

The application relates generally to the field of cancer therapy, in particular, to the immunotherapy of human cancer.

Background of the Invention

It has been found that inbred mice and rats can be immunized phrophylactically against tumors derived from mice and rats of the same genetic background (Gross (1943) Cancer Res. 3:323-326; Prehn et al. (1957) J. Natl. Cancer Inst. 18:769-778; Klein et al. (1960) Cancer Res. 20:1561-1572; Old et al. (1962) Ann NY Acad. Sci. 101:80-106; for review, see Srivastava et al. (1988) Immunology Today 9:78-83). These studies not only showed that mice vaccinated with inactivated cancer cells become immunized against subsequent challenges of live cancer cells but also demonstrated the existence of tumor-specific antigens.

Further studies revealed that the phenomenon of prophylactically induced immunity is tumor-specific. Although mice can be specifically immunized against the tumor cells that were used to immunize them they still remain sensitive to challenges with other unrelated tumors (Basombrio (1970) Cancer Res. 30:2458-2462, Globerson et al. (1964) J. Natl. Cancer Inst. 32:1229-1243). The demonstration of immunogenicity of cancer cells led to a search for the cancer-derived molecules which elicit resistance to tumor challenges. The

general approach was to fractionate cancer cell-derived proteins and test them individually for their ability to immunize mice against the cancers from which the fractions were prepared (see Srivastava et al. (1988) supra; Old (1981) Cancer Res. 41:361-375). A number of proteins have been identified by this method, however, a large proportion of these proteins are related to a class of proteins known as stress-induced proteins or stress proteins (Lindquist et al. (1988) Annual Rev. Genet. 22:631-677). Because the stress proteins are among the most highly conserved and abundant proteins in nature, they are unlikely candidates for tumor specific antigens. Stress proteins have subsequently been shown to non covalently associate with a variety of peptides thereby to form stress protein-peptide complexes (Gething et al. (1992) Nature 355:33-45; Lindquist et al. (1988) supra; Young (1990) Annu. Rev. Immunol. 8:401-420; Flynn et al. 1991) Nature 353:726-730).

Studies have also shown that stress protein-peptide complexes lose their immunogenicity upon treatment with ATP (Udono et al. (1993) J. Exp. Med. 178:1391-1396). This treatment is known to dissociate the stress-protein peptide complex into its stress protein and peptide components. Considering that there are no differences in the structure of stress proteins derived from normal and tumor cells, and that stress proteins bind a wide spectrum of peptides in an ATP dependent manner it appears that the antigenicity of the stress protein-peptide complex results not from the stress protein per se, but from the peptide associated with the stress protein.

One of the major conceptual difficulties in cancer immunotherapy has been the possibility that human cancers, like cancers of experimental animals, are antigenically distinct. Clearly, there is some recent evidence for existence of common tumor antigens (Kawakami et al. (1992) J. Immunol. 148:638-643; Darrow et al. (1989) J. Immunol. 142:3329-3334), and this augurs well for prospects of cancer immunotherapy. Nonetheless, in light of the overwhelming evidence from experimental and human systems, it is reasonable to assume that at the very least, human tumors would show tremendous antigenic diversity and heterogeneity.

The prospect of identification of the immunogenic antigens of individual tumors from cancer patients (or even of 'only' several different types of immunogenic antigens in case the antigens are shared), is daunting to the extent of being impractical. Conventional cancer therapies typically are based on the isolation and characterization of specific antigenic determinants which then may become the target for subsequent immunotherapies. In addition, although studies have demonstrated that mammals can be immunized prophylactically against tumors derived from mammals of the same genetic background, heretofore it has not been appreciated that a mammal harboring a tumor can be therapeutically immunized with a composition derived from its own tumor as a means of treating a cancer preexisting in the mammal.

Accordingly, it is an object of the instant invention to provide a novel method for therapeutically inhibiting proliferation of tumors in a mammal. The

method described herein does not require the isolation and characterization of specific antigenic determinants, and accordingly provides a more rapid approach for making and using immunogenic compositions effective in inhibiting the proliferation of specific predetermined tumors in mammals.

This and other objects and features of the invention will be apparent from the description and claims which follow.

Summary of the Invention

The observation that stress proteins chaperone the antigenic peptides of the cells from which they are derived provides an approach for readily isolating antigenic peptides for a preselected tumor. Once isolated, the stress protein-peptide complexes are administered back to the animal from which they were derived in order to elicit an immune response against a preexisting tumor. Accordingly, this approach circumvents the necessity of isolating and characterizing specific tumor antigens and enables the artisan to readily prepare immunogenic compositions effective against a preselected tumor.

In its broadest aspect, the invention provides a method for inhibiting proliferation of a preselected tumor in a mammal. The method comprises administering to the mammal undergoing therapy a composition comprising a pharmaceutically acceptable carrier in combination with a stress protein-peptide complex. The complex having been isolated from a tumor cell previously excised from the mammal and characterized in that it is operative to initiate in the mammal an immune response against the tumor cells from which it was derived. The complex subsequently is administered back to the mammal in an amount sufficient to elicit in the mammal an immune response against the tumor cells thereby to inhibit proliferation of any tumor cells still remaining in the mammal.

It is contemplated that this approach may be used in combination with other conventional cancer therapies

which include, for example, surgery, radiation therapy and chemotherapy. For example, following surgical excision of cancerous tissue the artisan, using the principles described herein, may isolate stress protein-peptide complexes from the excised tissue and administer the complex back to the mammal. The complex subsequently induces a specific immune response against any remaining tumor cells that were not excised during surgery. The approach is amenable to cancer therapy when the primary tumor has metastasized to different locations with the body.

The term "tumor" as used herein, is understood to mean any abnormal or uncontrolled growth of cells which may result in the invasion of normal tissues. It is contemplated also that the term embraces abnormal or uncontrolled cell growths that have metastasized, i.e., abnormal cells that have spread from a primary location in the body (i.e., primary tumor) to a secondary location spatially removed from the primary tumor.

The term "stress protein" as used herein, is understood to mean any cellular protein which satisfies the following criteria. It is a protein whose intracellular concentration increases when a cell is exposed to stressful stimuli, is capable of binding other proteins or peptides, and is capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) and/or low pH. Stressful stimuli include, but are not limited to, heat shock, nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens.

The first stress proteins to be identified were the heat shock proteins (Hsp's). As their name suggests, Hsp's typically are induced by a cell in response to heat shock. Three major families of mammalian Hsp's have been identified to date and include Hsp60, Hsp70 and Hsp90. The numbers reflect the approximate molecular weight of the stress proteins in kilodaltons. The members of each of the families are highly conserved, see for example, Bardwell et al. (1984) Proc. Natl. Acad. Sci. 81:848-852; Hickey et al. (1989) Mol. Cell Biol. 9:2615-2626; Jindal (1989) Mol. Cell. Biol. 9:2279-2283, the disclosures of which are incorporated herein by reference. Members of the mammalian Hsp90 family identified to date include cytosolic Hsp90 (also known as Hsp83) and the endoplasmic reticulum counterparts Hsp90 (also known as Hsp83), Hsp87, Grp94 (also known as ERp99) and gp96. See for example, Gething et al. (1992) Nature 355:33-45 the disclosure of which is incorporated herein by reference. Members of the Hsp70 family identified to date include: cytosolic Hsp70 (also known as p73) and Hsc70 (also known as p72); the endoplasmic reticulum counterpart BiP (also known as Grp78); and the mitochondrial counterpart Hsp 70 (also known as Grp75), Gething et al. (1992) supra. To date, members of the mammalian Hsp60 family have only been identified in the mitochondria, Gething et al. (1992) supra.

In addition, it has been discovered that the Hsp-60, Hsp-70 and Hsp-90 families are composed of proteins related to the stress proteins in amino acid sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stressful stimuli. Accordingly, it is contemplated

that the definition of stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with members of the three families whose expression levels in a cell are stimulated in response to stressful stimuli.

The term "peptide", as used herein, is understood to mean any amino acid sequence isolated from a mammalian tumor cell in the form of a stress protein-peptide complex.

The term "immunogenic stress protein-peptide complex", as used herein, is understood to mean any complex which can be isolated from a mammalian tumor cell and comprises a stress protein non covalently associated with a peptide. The complex is further characterized in that it is operative to induce in the mammal an immune response against the tumor cells from which the complex was derived.

The term "immune response" is understood to mean any cellular process that is produced in the mammal following stimulation with an antigen and is directed toward the elimination of the antigen from the mammal. The immune response typically is mediated by one or more populations of cells characterized as being lymphocytic and/or phagocytic in nature.

In a more specific aspect of the invention, the stress protein in the stress protein-peptide complex is selected from the group consisting of Hsp70, Hsp90 and gp96. Stress protein-peptide complexes which include

Hsp70-peptide, Hsp90-peptide and gp96-peptide complexes may be isolated simultaneously from a batch of tumor cells excised from a mammal. During immunotherapy it is contemplated that one or more of the aforementioned complexes may be administered to the mammal in order to stimulate the optimal immune response against the tumor.

It is contemplated that the method described herein is particularly useful in the treatment of human cancer. However, it is contemplated that the methods described herein likewise will be useful in immunotherapy of cancers in other mammals, for example, farm animals (i.e., cattle, horses, goats, sheep and pigs) and household pets (i.e., cats and dogs).

In another aspect of the invention, it is contemplated that the immune response is effected by means of a T cell cascade, and more specifically by means of a cytotoxic T cell cascade. The term "cytotoxic T cell", as used herein, is understood to mean any T lymphocyte expressing the cell surface glycoprotein marker CD8 that is capable of targeting and lysing a target cell which bears a class I histocompatibility complex on its cell surface and is infected with an intracellular pathogen.

In another aspect of the invention, the stress protein-peptide complexes may be administered to the mammal in combination with a therapeutically active amount of a cytokine. As used herein, the term "cytokine" is meant to mean any secreted polypeptide that influences the function of other cells mediating an immune response. Accordingly, it is contemplated

that the complex can be coadministered with a cytokine to enhance the immune response directed against the tumor. Preferred cytokines include, but are not limited to, interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon α (IFNα), interferon β (IFNβ), interferon γ (IFNγ), tumor necrosis factor α (TNFα), tumor necrosis factor β (TNFβ), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor β (TGF-β).

The complex may be administered to a mammal when combined with a conventional pharmaceutically acceptable carrier, adjuvant, or excipient using techniques well known in the art. The dosage and means of administration of the family of stress protein-peptide complexes necessarily will depend upon a variety of factors such as the stability of the complex under physiological conditions, the effectiveness of the complex at eliciting an immune response, the size and distribution of the tumor, and the age, sex and weight of the mammal undergoing therapy.

Typically, the complex should be administered in an amount sufficient to initiate in the mammal an immune response against the tumor from which the complex was derived and in an amount sufficient to inhibit proliferation of the tumor cells in the mammal. The amount of stress protein-peptide complex administered

preferably is in the range of about 1-1000 micrograms of complex/kg body weight of the mammal/administration, and most preferably about 100-250 micrograms of complex/kg body weight of the mammal/administration. It is contemplated that typical dose will be in the range of about 5 to about 20mg for a human subject weighing about 75 kg. In addition, it is contemplated that the strength of the immune response may be enhanced by repeatedly administering the complex to the individual. The mammal preferably receives at least two doses of the stress protein-peptide complex at weekly intervals. If necessary, the immune response may be boosted at a later date by subsequent administration of the complex. It is contemplated, however, that the optimal dosage and immunization regimen may be found by routine experimentation by one skilled in the art.

Detailed Description.

The invention is based on the observation that stress protein-peptide complexes chaperone antigenic peptides of the cells from which they are derived. Conventional cancer therapies are based upon the isolation an characterization of tumor specific antigens which then become the target for a specific therapeutic regime. Because of the antigenic diversity of mammalian cancers the isolation and characterization of specific tumor antigens for each specific tumor can be impractical. The instant invention thus provides an alternative approach to cancer immunotherapy by obviating the necessity of isolating and characterizing tumor specific antigens for each tumor being treated.

The invention described herein provides a method for inhibiting proliferation of a preselected tumor in a mammal. The method comprises isolating or obtaining tumor cells from the mammal undergoing therapy. This is accomplished readily using conventional surgical procedures well known in the art. Typically, tumor cells are excised from the mammal during routine surgical recision of the tumor. The method then involves isolating stress protein-peptide complexes from the excised tumor cells. This is accomplished using any one of the isolation procedures described in detail herein below. The stress protein-peptide complexes are characterized in that when they are administered back to the mammal they are capable of initiating a specific immune response against the same type of tumor cells that they were derived from. Finally, the method comprises the step of administering back to the mammal the isolated stress protein-peptide complex in an amount sufficient to elicit in the mammal an immune response against the tumor cells thereby

inhibiting proliferation of any tumor cells remaining in the mammal.

It is contemplated that this approach may be used in combination with one or more conventional cancer therapies which include, for example, surgery, radiation therapy and chemotherapy. For example, following surgical excision of cancerous tissue the artisan, using the principles described herein, may isolate stress protein-peptide complexes from the excised tissue and administer the complex back to the mammal. The complex then induces in the mammal a specific immune response against any tumor cells that were not removed during surgery. Alternatively, the method described herein provides a novel approach for treating cancer when the primary tumor has metastasized to multiple locations with the body. For example, when the cancer has metastasized, making surgical intervention impractical, a stress protein-peptide complex may be used either alone or in combination with another standard chemotherapeutic agent in the treatment of the cancer.

It is contemplated that the invention has particular utility in the immunotherapy of human cancer, however, it is appreciated that the methodologies described herein may be applied to the treatment of cancers occurring in, for example, farm animals (i.e., cattle, horses, sheep, goats and pigs) and household pets (i.e., cats and dogs).

The main advantage this approach has over conventional methodologies is that it is not necessary to isolate and characterize the tumor specific antigen for each tumor. Once the stress protein-peptide complex has been isolated it is simply administered back the mammal without further characterization. Since the procedures for isolating the immunogenic complexes are routine and well known in the art, the artisan may rapidly and routinely prepare a specific immunogenic composition "tailor-made" for each individual being treated.

Another advantage of the instant method over previous methodologies is that the administration of purified stress protein-peptide complexes back to the individual from which they were derived eliminates the risk of inoculating the mammal undergoing therapy with potentially transforming agents (i.e., transforming DNA) and/or immunosuppressive agents which can be an issue when the complex is present in a biochemically undefined tumor or tumor extract. In addition, stress protein-peptide complexes can induce significant tumor immunity in the absence of adjuvants. Accordingly, while adjuvants may further enhance the immunotherapeutic properties of the complex, their availability is not a pre-condition for inducing a significant immune response.

It is contemplated that this method can be used in the treatment of a variety of tumors, for example, tumors that are mesenchymal in origin (sarcomas) i.e., fibrosarcomas; myxosarcomas; liposarcomas; chondrosarcomas; osteogenic sarcomas; angiosarcomas; endotheliosarcomas; lymphangiosarcomas;

synoviosarcomas; mesotheliosarcomas; Ewing's tumors; myelogenous leukemias; monocytic leukemias; malignant lymphomas; lymphocytic leukemias; plasmacytomas; leiomyosarcomas and rhabdomyosarcoma.

In addition, it is contemplated that this method can be used in the treatment of tumors that are epithelial in origin (carcinomas) i.e., squamous cell or epidermal carcinomas; basal cell carcinomas; sweat gland carcinomas; sebaceous gland carcinomas; adenocarcinomas; papillary carcinomas; papillary adenocarcinomas; cystadenocarcinomas; medullary carcinomas; undifferentiated carcinomas (simplex carcinomas); bronchogenic carcinomas; bronchial carcinomas; melanocarcinomas; renal cell carcinomas; hepatocellular carcinomas; bile duct carcinomas; papillary carcinomas; transitional cell carcinomas; squamous cell carcinomas; choriocarcinomas; seminomas; embryonal carcinomas malignant teratomas and teratocarcinomas. Generic methodologies useful in the preparation of compositions effective at inducing an immune response against these tumors are discussed in detail herein below.

Although not wishing to be bound by theory, it is contemplated that the stress protein-peptide complexes stimulate an immune response against the tumor cells from which they are derived by means of a T cell cascade. Previous experiments have demonstrated that mice immunized prophylactically with stress protein-peptide preparations derived from a tumor originating in the same strain of mouse or rat develop immunological resistance to the tumor from which it was isolated. The mice, however, fail to develop immunity

against antigenically distinct tumors. Furthermore, stress protein-peptide complexes derived from normal tissues do not elicit resistance to any tumors tested. See for example, Srivastava et al. (1984) Int. J. Cancer 33:417; Srivastava et al. (1986) Proc. Natl. Acad. Sci. USA 83:3407; Palladino et al. (1987) Cancer Res. 47:5074; Feldweg et al. (1993) J. Cell Biochem. Suppl. 17D:108 (Abst.); Udono et al. (1993) J. Cell. Biochem. Suppl. 17D:113 and Udono (1993) J. Exp. Med.178:1391-1396, the disclosures of which are incorporated herein by reference. Recently, it has been established prophylactic immunity typically is mediated by means of a T cell cascade, more specifically by means of a cytotoxic T cell cascade. See for example, Blachere et al. (1993) J. Immunother. 14:352-356, the disclosure of which is incorporated by reference herein. Accordingly, it is contemplated that the stress-protein complexes may also mediate their effect therapeutically by a similar mechanism; specifically, via a cytotoxic T cell cascade.

It is contemplated that the stress protein-peptide complexes typically will be isolated directly from tumor tissue excised from the mammal being treated. Under certain conditions, however, the amount of tumor tissue available for isolation of the complex may be limiting. Accordingly, it is contemplated that the excised tumor tissue may be proliferated using techniques well known in the art prior to the isolation of the stress protein-peptide complexes. For example, the excised tumor tissue may be proliferated either in vivo, for example, by transfecting a nude mouse with a sample of the tumor tissue, or in vitro, for example, by serially passaging the tumor cells in culture. The

proliferated tumor tissue subsequently can be harvested and used as a starting material for the isolation of the stress protein-peptide complex.

Stress proteins useful in the practice of the instant invention may be defined as any cellular protein that satisfies the following criteria. It is a protein whose intracellular concentration increases when a cell is exposed to a stressful stimuli, is capable of binding other proteins or peptides, and is capable of releasing the bound proteins or peptides in the presence of adenosine triphosphate (ATP) or low pH.

The first stress proteins to be identified were the Hsp's which are synthesized in a cell in response to heat shock. To date, three major families of mammalian Hsp's have been identified and include Hsp60, Hsp70 and Hsp90 where the numbers reflect the approximate molecular weight of the stress proteins in kilodaltons. Many members of these families were found subsequently to be induced in response to other stressful stimuli including, but not limited to, nutrient deprivation, metabolic disruption, oxygen radicals, and infection with intracellular pathogens. See for example: Welch (May 1993) Scientific American 56-64; Young (1990) supra; Craig (1993) Science 260:1902-1903; Gething et al (1992) supra; and Lindquist et al. (1988) supra, the disclosures of which are incorporated herein by reference. It is contemplated that mammalian stress proteins belonging to all three families may be useful in the practice of the instant invention.

The major stress proteins accumulate to very high levels in stressed cells but occur at low to moderate

levels in cells that have not been stressed. For example, the highly inducible mammalian Hsp70 is hardly detectable at normal temperatures but becomes one of the most actively synthesized proteins in the cell upon heat shock (Welch et al. (1985), J. Cell. Biol. 101:1198-1211). In contrast, Hsp90 and Hsp60 proteins are abundant at normal temperatures in most, but not all, mammalian cells and are further induced by heat (Lai et al. (1984), Mol. Cell. Biol. 4:2802-10; van Bergen en Henegouwen et al. (1987), Genes Dev., 1:525-31).

Members of the mammalian Hsp90 family identified to date include cytosolic Hsp90 (also known as Hsp83) and the endoplasmic reticulum counterparts Hsp90 (also known as Hsp83), Hsp87, Grp94 (also known as ERp99) and gp96 (Gething et al. (1992) supra). Members of the Hsp70 family identified to date include: cytosolic Hsp70 (also known as p73) and Hsc70 (also known as p72), the endoplasmic reticulum counterpart BiP (also known as Grp78) and the mitochondrial counterpart Hsp 70 (also known as Grp75), Gething et al. (1992) supra. To date, members of the mammalian Hsp60 family have only been identified in the mitochondria, Gething et al. (1992) supra.

Stress proteins are among the most highly conserved proteins in existence. For example, DnaK, the Hsp70 from E. coli has about 50% amino acid sequence identity with Hsp70 proteins from eukaryotes (Bardwell et al. (1984) Proc. Natl. Acad. Sci. 81:848-852). The Hsp60 and Hsp90 families similarly exhibit high levels of intrafamilial conservation (Hickey et al. (1989) Mol. Cell Biol. 9:2615-2626; Jindal (1989) Mol. Cell. Biol.

9:2279-2283). In addition, it has been discovered that the Hsp60, Hsp70 and Hsp90 families are composed of proteins that are related to the stress proteins in sequence, for example, having greater than 35% amino acid identity, but whose expression levels are not altered by stress. Therefore it is contemplated that the definition of stress protein, as used herein, embraces other proteins, muteins, analogs, and variants thereof having at least 35% to 55%, preferably 55% to 75%, and most preferably 75% to 85% amino acid identity with members of the three families whose expression levels in a cell are enhanced in response to a stressful stimulus.

The immunogenic stress protein-peptide complexes of the invention may include any complex containing a stress protein non covalently associated with a peptide that is capable of inducing an immune response in a mammal. Preferred complexes include, but are not limited to, Hsp70-peptide, Hsp90-peptide and gp96-peptide complexes. For example, the mammalian stress protein gp96 which is the endoplasmic reticulum counterpart of the cytosolic Hsp90 may be used in the practice of the instant invention.

Typical procedures for isolating stress protein-peptide complexes useful in the practice of the instant invention are set forth in detail below.

Purification of Hsp70-peptide complexes.

The purification of Hsp70-peptide complexes has been described previously, see for example, Udono et al. (1993) supra.

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 min and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with phosphate buffered saline (PBS) containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1 mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 min. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated in 20mM Tris-Acetate pH 7.5, 20 mM NaCl, 0.1mM EDTA and 15mM 2-mercaptoethanol. The column is then developed with a 20 mM to 500 mM NaCl gradient and the eluted

fractions fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and characterized by immunoblotting using an appropriate anti-Hsp70 antibody (such as from clone N27F3-4, from StressGen).

Fractions strongly immunoreactive with the anti-Hsp70 antibody are pooled and the Hsp70-peptide complexes precipitated with ammonium sulfate; specifically with a 50%-70% ammonium sulfate cut. The resulting precipitate is then harvested by centrifugation at 17,000 rpm (SS34 Sorvall rotor) and washed with 70% ammonium sulfate. The washed precipitate is then solubilized and any residual ammonium sulfate removed by gel filtration on a Sephadex^{R} G25 column (Pharmacia).

The Hsp70-peptide complex can be purified to apparent homogeneity using this method. Typically 1mg of Hsp70-peptide complex can be purified from 1g of cells/tissue.

Purification of Hsp90-peptide complexes.

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 min and then

homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and then mixed with Con A Sepharose equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The material that fails to bind is harvested and dialyzed for 36 hours (three times, 100 volumes each time) against 10mM Tris-Acetate pH 7.5, 0.1 mM EDTA, 10mM NaCl, 1mM PMSF. Then the dialyzate is centrifuged at 17,000 rpm (Sorvall SS34 rotor) for 20 min. Then the resulting supernatant is harvested and applied to a Mono Q FPLC column equilibrated equilibrated with lysis buffer. The proteins are then eluted with a a salt gradient of 200mM to 600mM NaCl.

The eluted fractions are fractionated by SDS-PAGE and fractions containing the Hsp90-peptide complexes identified by immunoblotting using a anti-Hsp90 antibody such as 3G3 (Affinity Bioreagents). Hsp90-peptide complexes can be purified to apparent homogeneity using this procedure. Typically, 150-200 μg of Hsp90-peptide complex can be purified from 1g of cells/tissue.

Purification of gp96-peptide complexes.

Initially, tumor cells are suspended in 3 volumes of 1X Lysis buffer consisting of 5mM sodium phosphate buffer (pH7), 150mM NaCl, 2mM $CaCl_2$, 2mM $MgCl_2$ and 1mM phenyl methyl sulfonyl fluoride (PMSF). Then, the pellet is sonicated, on ice, until >99% cells are lysed as determined by microscopic examination. As an alternative to sonication, the cells may be lysed by mechanical shearing and in this approach the cells typically are resuspended in 30mM sodium bicarbonate pH 7.5, 1mM PMSF, incubated on ice for 20 min and then homogenized in a dounce homogenizer until >95% cells are lysed.

Then the lysate is centrifuged at 1000g for 10 minutes to remove unbroken cells, nuclei and other cellular debris. The resulting supernatant is recentrifuged at 100,000g for 90 minutes, the supernatant harvested and mixed with Con A Sepharose slurry equilibrated with PBS containing 2mM Ca^{2+} and 2mM Mg^{2+}. When the cells are lysed by mechanical shearing the supernatant is diluted with an equal volume of 2X Lysis buffer prior to mixing with Con A Sepharose. The supernatant is then allowed to bind to the Con A Sepharose for 2-3 hours at 4°C. The slurry is then packed into a column and washed with 1X lysis buffer until the OD_{280} drops to baseline. Then the column is washed with 1/2 column bed volume of 10% α-methyl mannoside (α-MM), the column sealed with parafilm and incubated at 37°C for 15 min. The column is then cooled to room temperature, the parafilm removed from the bottom of the column, and five column volumes of a α-MM is applied to the column. The eluate

is then fractionated and characterized by SDS-PAGE. Typically, the resulting gp96-peptide complex is about 60 to 95% pure depending upon the cell type and the tissue to lysis buffer ratio used.

If further purification is required, the sample can be applied to a Mono Q FPLC column equilibrated with a buffer containing 5mM sodium phosphate, pH7. The proteins are then eluted from the column with a 0-1M NaCl gradient. The gp96 fraction elutes between 400mM and 550mM NaCl.

As an alternative procedure, the gp96 fraction isolated from the 100,000g pellet can be resuspended in 5 volumes of PBS containing 1% sodium deoxycholate (without Ca^{2+} and Mg^{2+}) and incubated on ice for 1 h. The resulting suspension is centrifuged for 30 min at 20,000g and the resulting supernatant harvested and dialyzed against several changes of PBS (without Ca^{2+} and Mg^{2+}) to remove the detergent. The resulting dialysate is centrifuged for 90 min at 100,000g and the supernatant purified further. Then calcium and magnesium are both added to the supernatant to give final concentrations of 2mM. Then the sample is applied to a Mono Q HPLC column equilibrated with a buffer containing 5mM sodium phosphate, pH7 and the proteins eluted with a 0-1M NaCl gradient. The gp96 fraction elutes between 400mM and 550mM NaCl.

The gp96-peptide complexes can be purified to apparent homogeneity using this procedure. Typically about 10-20 μg of gp96 can be isolated from 1g cells/tissue using this method.

Formulation and Administration of the Complexes.

Once stress protein-peptide complexes have been purified from the excised tumor they are administered back to the mammal undergoing therapy in order to stimulate in the mammal an immune response against tumor cells from which the complex was derived. The stress protein-peptide complexes of the invention may either be stored or prepared for administration by mixing with physiologically acceptable carriers, excipients, or stabilizers. These materials should be non-toxic to the intended recipient at dosages and concentrations employed.

When the complex is water soluble it may be formulated in an appropriate buffer, for example PBS (5mM sodium phosphate, 150 mM NaCl, pH7.1) or other physiologically compatible solutions. Alternatively, if the resulting complex has poor solubility in aqueous solvents then it may be formulated with a non-ionic surfactant such as Tween, or polyethylene glycol.

Useful solutions for oral or parenteral administration may be prepared by any of the methods well known in the pharmaceutical art, described, for example, in Remington's Pharmaceutical Sciences, (Gennaro, A., ed.), Mack Pub., 1990. Formulations may include, for example, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. Formulations for direct administration, in particular, may include glycerol and other compositions of high viscosity. Biocompatible, preferably bioresorbable polymers, including, for example, hyaluronic acid, collagen,

tricalcium phosphate, polybutyrate, polylactide, polyglycolide and lactide/glycolide copolymers, may be useful excipients to control the release of the stress protein-peptide complexes in vivo.

Formulations for inhalation may contain as excipients, for example, lactose. Aqueous solutions may contain, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate. Oily solutions may be useful administration in the form of nasal drops. Gels may be applied topically intranasally.

The compounds provided herein can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable nontoxic excipients and carriers. In addition the formulations may optionally contain one or more adjuvants. Preferred adjuvants include, but are not limited to, pluronic tri-block copolymers, muramyl dipeptide and its derivatives, detoxified endotoxin, saponin and its derivatives such as QS-21 and liposomes. The present invention further envisages sustained release formulations in which the complex is released over an extended period of time.

The mode of administration of the family of stress protein-peptide complexes prepared in accordance with the invention will necessarily depend upon the stability of the complex under physiological conditions, and the size and distribution of the tumor within the mammal being treated. The preferred dosage of complex to be administered also is likely to depend on such variables as the size and distribution of the tumor, the age, sex and weight of the intended

recipient, the overall health status of the particular recipient, the relative biological efficacy of the complex, the formulation for the complex, the presence and types of excipients in the formulation, and the route of administration.

In general terms, the compounds of this invention may be provided in an aqueous physiological buffer solution containing about 0.001 to 10% w/v compound for parenteral administration. Preferred dosages range from about 1 to about 1000 micrograms of complex/kg body weight of recipient/administration and most preferably range from about 100 to about 250 micrograms of complex/kg body weight of recipient/administration. In particular, it is contemplated that a typical dose will range from about 5mg to about 20mg for a human subject weighing about 75kg. These quantities, however, may vary according to the adjuvant coadministered with the complex.

The complex preferably comprises part of an aqueous solution which may be administered using standard procedures, for example, intravenously, subcutaneously, intramuscularly, intraorbitally, ophthalmically, intraventricularly, intracranially, intracapsularly, intraspinally, intracisternally, intraperitoneally, buccal, rectally, vaginally, intranasally or by aerosol administration. The aqueous solution preferably is physiologically acceptable so that in addition to delivery of the desired complex to the mammal, the solution does not otherwise adversely affect the mammal's electrolyte and/or volume balance. The aqueous medium for the complex thus may comprise normal physiologic saline (0.9% NaCl, 0.15M), pH 7-7.4 or

other pharmaceutically acceptable salts thereof.

Preferably the recipient should be vaccinated three times at two week intervals. If necessary, the responses may be boosted at a later date by subsequent administration of the complex. It is contemplated that the optimal dosage and vaccination schedule may be determined empirically for each stress protein-peptide complex using techniques well known in the art.

Various cytokines, antibiotics, and other bioactive agents also may be coadministered with the stress protein-peptide complexes. For example, various known cytokines, i.e., interleukin-1α (IL-1α), interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon α (IFNα), interferon β (IFNβ), interferon γ (IFNγ), tumor necrosis factor α (TNFα), tumor necrosis factor β (TNFβ), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor β (TGF-β) may be coadministered with the complexes in order to maximize the physiological response. However, it is anticipated that other but as yet undiscovered cytokines may be effective in the invention. In addition, conventional antibiotics may be coadministered with the stress protein-peptide complex. The choice of suitable antibiotics will however be dependent upon the disease in question.

Example I

In this example, C57BL/6 and C3H mice approximately 100 g in weight, are purchased from Jackson Laboratories, Bar Harbor, Me. Malignant tumor cells are then injected subcutaneously into mice in order to induce experimental tumors in the mice. Specifically, malignant spindle cell carcinoma 6139 cells are injected subcutaneously into the C3H mice, malignant mouse Lewis lung carcinoma cells are injected subcutaneously into C57BL/6 mice and malignant mouse B16 melanoma cells are injected subcutaneously into C57BL/6 mice.

When the tumors have grown to a size such that they are both visible and palpable, a sample of the tumor tissue is excised. As a control, normal non malignant tissue is excised from some mice bearing the experimental tumors.

Then gp96-peptide, Hsp90-peptide and Hsp70-peptide complexes are isolated from both the excised normal and tumor derived tissues using the methods described hereinabove. Once isolated, the complexes are combined with PBS and administered back to the mice from which the complexes were derived. Usually 6 mice are tested in each experiment. The experiments are performed using the schedule set forth below:

Experiment	Composition administered back to mice
1	gp96-peptide
2	Hsp70-peptide
3	Hsp90-peptide
4	gp96-peptide and Hsp70-peptide
5	gp96-peptide and Hsp90-peptide
6	Hsp70-peptide and Hsp90-peptide
7	Hsp70-peptide, Hsp90-peptide and gp96-peptide
8	buffer alone

In one series of experiments the complexes are isolated from tumor cells whereas in a second series the complexes are isolated from normal cells. The mice are inoculated three times at weekly intervals with 20 micrograms (total weight) of the preselected complex(es). During therapy, the size of each tumor is measured daily. After 4 weeks the mice are sacrificed and the development of the tumor examined histologically. In addition, the sacrificed mice are examined for the presence or absence of metastasis.

It is expected that the tumors in mice treated with complexes derived from normal tissue will continue to grow and metastasize. In contrast, it is expected that the tumors in the mice treated with the complexes derived from the tumor tissue will be exhibit slower growth than the tumors in the control animals, and in some cases, it is expected that the tumor mass may get smaller and the tumor exhibit remission during therapy.

Other Embodiments

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

1. A method for inhibiting proliferation of a tumor in a mammal, the method comprising:

administering to the mammal harboring the tumor a composition comprising,

(a) an immunogenic stress protein-peptide complex isolated from a cell derived from the tumor, said complex being operative to initiate in the mammal an immune response against said tumor, and

(b) a pharmaceutically acceptable carrier,

in an amount sufficient to elicit in the mammal an immune response against the tumor thereby inhibiting proliferation of the tumor.

2. The method of claim 1, wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

3. The method of claim 1, wherein a peptide in the complex is non covalently associated with the stress protein.

4. The method of claim 1, wherein administering the complex initiates an immune response mediated by a T cell.

5. The method of claim 4, wherein administering the complex initiates an immune response mediated by a cytotoxic T cell.

6. The method of claim 1, wherein the complex is administered to the mammal in an amount in the range of about 1 to about 1000 micrograms of complex/kg body weight of mammal/administration.

7. The method of claim 6, wherein said amount is in the range of about 100 to about 250 micrograms of complex/kg body weight of mammal/administration.

8. The method of claim 1, wherein the complex is administered repeatedly to the mammal.

9. The method of claim 1, wherein the composition is administered to the mammal in combination with a cytokine.

10. A method for inhibiting proliferation of a tumor in a mammal, the method comprising the steps of:

(a) providing a tumor cell excised from the mammal,

(b) isolating from the cell an immunogenic stress protein-peptide complex operative to initiate in the mammal an immune response against the tumor cell, and

(c) administering to the mammal the isolated stress protein-peptide complex in an amount sufficient to elicit in the mammal an immune response against the tumor cell thereby to inhibit proliferation of any tumor cell remaining in the mammal.

11. The method of claim 10, wherein the stress protein in the complex is a Hsp70, a Hsp90 or a gp96.

12. The method of claim 10, wherein a peptide in the complex is non covalently associated with the stress protein.

13. The method of claim 10, wherein administering the complex initiates an immune response mediated by a T cell.

14. The method of claim 13, wherein administering the complex initiates an immune response mediated by a cytotoxic T cell.

15. The method of claim 10, wherein the complex is administered to the mammal in an amount in the range of about 1 to about 1000 micrograms of complex/kg body weight of mammal/administration.

16. The method of claim 15, wherein said amount is in the range of about 100 to about 250 micrograms of complex/kg body weight of mammal/administration.

17. The method of claim 10, wherein the complex is administered repeatedly to the mammal.

18. The method of claim 10, wherein said complex is administered to the mammal in combination with a cytokine.

Abstract of the Disclosure

Disclosed is a method for inhibiting the proliferation of a tumor in a mammal. The method involves the steps of (a) isolating a stress protein-peptide complex from tumor cells previously removed from the mammal and (b) administering the isolated stress protein-peptide complex back to the mammal in order to stimulate in the mammal an immune response against the tumor from which the complex was isolated. Stress protein-peptide complexes having particular utility in the practice of the instant invention include the Hsp70-peptide, Hsp90-peptide and gp96-peptide complexes.

COMBINED DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION

(Original, Design, National Stage of PCT, Supplemental, Divisional, Continuation or CIP)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type: (check one applicable item below)

_X_ original

___ design

___ supplemental

___ national stage of PCT

___ divisional

___ continuation

___ continuation-in-part (CIP)

INVENTORSHIP IDENTIFICATION

My residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

IMMUNOTHERAPEUTIC HEAT SHOCK PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER

SPECIFICATION IDENTIFICATION

the specification of which (check one):

___ is attached hereto.

_X_ was filed on September 30, 1994 as Application Serial No. 08/315,892 or

___ Express Mail No., as Serial No. not yet known _____ and was amended on _____ (if applicable).

___ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____ (if any).

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

___ In compliance with this duty there is attached an information disclosure statement. 37 CFR 1.97.

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Check one:

_X_ no such applications have been filed.

___ such applications have been filed as follows:

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (mo.,day, year)	Priority Claimed Under 37 USC 119
			___ YES NO ___
			___ YES NO ___
			___ YES NO ___

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 USC 120:

U.S. APPLICATIONS	U.S. FILING DATE	STATUS
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, aband.)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, aband.)

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the United States Patent and Trademark Office connected therewith:

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Direct telephone calls to:

Edmund R. Pitcher (617) 248-7589
(name and telephone number)

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

Pramod K. Srivastava
Full name of sole or first inventor

India
Citizenship

P K Srivastava
Inventor's signature

Dec 8' 94
Date

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Post Office Address

CHECK PROPER BOX(ES) FOR ANY OF THE FOLLOWING ADDED PAGE(S) WHICH FORM A PART OF THIS DECLARATION

___ Signature for third and subsequent joint inventors. Number of pages added ____.

___ Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. Number of pages added ____.

___ Signature for inventor who refuses to sign or cannot be reached by person authorized under 37 CFR 1.47. Number of pages added ____.

___ Added pages to combined declaration and power of attorney for divisional, continuation, or continuation in-part (CIP) application. ___ Number of pages added.

___ Authorization of attorney(s) to accept and follow instructions from representative.

If no further pages form a part of this Declaration then end this Declaration with this page and check the following item.

_X_ This declaration ends with this page.

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:
Pramod K. Srivastava

Serial No.: 08/315,892

Group Art Unit: 1205

Filed: September 30, 1994

Examiner:

For: IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER

Pennie & Edmonds
1155 Avenue of the Americas
New York, New York 10036

Attorney Docket No.: 8449-008

REVOCATION AND POWER OF ATTORNEY

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231

S I R :

Pramod K. Srivastava, residing at 3805 Greystone Avenue, Riverdale, New York 10463, revokes all previous powers of attorney and appoints S. Leslie Misrock (Reg. No. 18872), Harry C. Jones, III (Reg. No. 20280), Berj A. Terzian (Reg. No. 20060), Gerald J. Flintoft (Reg. No. 20823), David Weild, III (Reg. No. 21094), Jonathan A. Marshall (Reg. No. 24614), Joseph V. Colaianni (Reg. No. 20019), Charles E. McKenney (Reg. No. 22795), Philip T. Shannon (Reg. No. 24278), Barry D. Rein (Reg. No. 22411), Stanton T. Lawrence, III (Reg. No. 25736), Francis E. Morris (Reg. No. 24615), Charles E. Miller (Reg. No. 24576), Gidon D. Stern (Reg. No. 27469),

EXPRESS MAIL CERTIFICATION

"Express Mail" label No. TB686 622 783US Date of Deposit April 5, 1995

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

MICHAEL VIGUIE

(Type or print name of person mailing paper or fee)

(Signature of person mailing paper or fee)

John J. Lauter, Jr. (Reg. No. 27814), Brian M. Poissant (Reg. No. 28462), Brian D. Coggio (Reg. No. 27624), Rory J. Radding (Reg. No. 28749), Stephen J. Harbulak (Reg. No. 29166), Donald J. Goodell (Reg. No. 19766), James N. Palik (Reg. No. 25510), Thomas E. Friebel (Reg. No. 29258), Laura A. Coruzzi (Reg. No. 30742), Jennifer Gordon (Reg. No. 30753), Jon R. Stark (Reg. No. 30111), Allan A. Fanucci (Reg. No. 30256), Geraldine F. Baldwin (Reg. No. 31232), Victor N. Balancia (Reg. No. 31231), Albert P. Halluin (Reg. No. 25227) and Marcia H. Sundeen (Reg. No. 30893), whose address is Pennie & Edmonds, 1155 Avenue of the Americas, New York, New York 10036, and each of them his attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith.

Please direct all correspondence to Pennie & Edmonds, located at 1155 Avenue of the Americas, New York, New York 10036, and direct all telephone calls to Pennie & Edmonds at (212) 790-9090.

Dated: 6/3/05 By: _____
PRAMOD K. SRIVASTAVA

SUPPLEMENTAL DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

IMMUNOTHERAPEUTIC STRESS PROTEIN-PEPTIDE COMPLEXES AGAINST CANCER

and for which a patent application:
is attached hereto and includes amendment(s) filed on _____ *(if applicable)*
☒ was filed in the United States on September 30, 1994 as Application Serial No. 08/315,892 *(for declaration not accompanying application)*
with amendment(s) filed on January 29, 1996, October 8, 1996 and September 17, 1997 *(if applicable)*
☐ was filed as PCT international application Serial No. _____ on _____ and was amended under PCT Article 34 on February 23, 1996

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED
08/180,685	January 13, 1994		X	

POWER OF ATTORNEY: As a named inventor, I hereby appoint S. Leslie Misrock (Reg. No. 18872), Harry C. Jones, III (Reg. No. 20280), Berj A. Terzian (Reg. No. 20060), Gerald J. Flintoft (Reg. No. 20823), David Weild, III (Reg. No. 21094), Jonathan A. Marshall (Reg. No. 24614), Barry D. Rein (Reg. No. 22411), Stanton T. Lawrence, III (Reg. No. 25736), Isaac Jarkovsky (Reg. No. 22713), Joseph V. Colaianni (Reg. No. 20019), Charles E. McKenney (Reg. No. 22795), Philip T. Shannon (Reg. No. 24278), Francis E. Morris (Reg. No. 24615), Charles E. Miller (Reg. No. 24576), Gidon D. Stern (Reg. No. 27469), John J. Lauter, Jr. (Reg. No. 27814), Brian M. Poissant (Reg. No. 28462), Brian D. Coggio (Reg. No. 27624), Rory J. Radding (Reg. No. 28749), Stephen J. Harbulak (Reg. No. 29166), Donald J. Goodell (Reg. No. 19766), James N. Palik (Reg. No. 25510), Thomas E. Friebel (Reg. No. 29258), Laura A. Coruzzi (Reg. No. 30742), Jennifer Gordon (Reg. No. 30753), Jon R. Stark (Reg. No. 30111), Allan A. Fanucci (Reg. No. 30256), Geraldine F. Baldwin (Reg. No. 31232), Victor N. Balancia (Reg. No. 31231), Albert P. Halluin (Reg. No. 25227), Samuel B. Abrams (Reg. No. 30605), Steven L. Wallach (Reg. No. 35402), Marcia H. Sundeen (Reg. No. 30893), Paul J. Zegger (Reg. No. 33821), Edmond R. Bannon (Reg. No. 32110), Bruce J. Barker (Reg. No. 33291), Adriane M. Antler (Reg. No. 32605), Ann L. Gisolfi (Reg. No. 31956), SaraLynn Mandel (Reg. No. 31853), Mark A. Farley (Reg. No. 33170), James G. Markey (Reg. No. 31636), and Charles F. Hoyng (Reg. No. 35548), all of Pennie & Edmonds LLP, whose addresses are 1155 Avenue of the Americas, New York, New York 10036, 1667 K Street N.W., Washington, DC 20006 and 2730 Sand Hill Road, Menlo Park, CA 94025, and each of them, my attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith.

SEND CORRESPONDENCE TO:	PENNIE & EDMONDS 1155 AVENUE OF THE AMERICAS NEW YORK, N.Y. 10036-2711	DIRECT TELEPHONE CALLS TO: PENNIE & EDMONDS DOCKETING (212) 790-2803	

201	FULL NAME OF INVENTOR	LAST NAME Srivastava	FIRST NAME Pramod	MIDDLE NAME K.	
	RESIDENCE & CITIZENSHIP	CITY Avon	STATE OR FOREIGN COUNTRY Connecticut	COUNTRY OF CITIZENSHIP INDIA	
	POST OFFICE ADDRESS	STREET 70 Pheasant Run	CITY Avon	STATE OR COUNTRY Connecticut	ZIP CODE 06030

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
[signature] Pramod K. Srivastava		
DATE 12/11/97	DATE	DATE